Laboratory Methods for "Struewing JP, Hartge P, Wacholder S, Baker SM, Berlin M, McAdams M, Timmerman MM, Brody LC, Tucker MA. The risk of cancer associated with the specific mutations of *BRCA1* and *BRCA2* among Ashkenazi Jews. New Engl J Med 1997;336:1401-8."
Also available on the WWW at http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/Methods/nejm97.html

Phlebotomists used a fingerstick procedure (Tenderlet, ITC Corp, Edison, NJ) to collect 100 to 150 microliters of blood into three heparinized capillary tubes. The blood was transferred onto collection cards (Isocode, Schleicher & Scheull, Keene, NH) which were air dried, sealed in plastic bags with desiccant, and mailed to the laboratory at room temperature.

DNA was isolated from two 3-mm punches of a blood spot in 96-well trays according to manufacturer's instructions, with slight modification. Clean chromatography paper was punched four to six times between samples to reduce carryover. The blood spots were washed twice in 200 microliters of DEPC-treated water, 100 microliters of water were then added to each tube and heated to 95^{o}C for 30 minutes, pulse vortexing 15 times after 15 minutes and 60 times after 30 minutes. To monitor for contamination, one well of each 96-well tray contained only collection paper (without blood), and three wells were processed with only reagents.

Samples were analyzed for specific *BRCA1* and *BRCA2* mutations using PCR-based assays. Allele specific oligonucleotide (ASO) assays were used for the *BRCA1* 185delAG and 188del11 mutations and allele specific PCR's were used for the *BRCA1* 5382insC mutation and the *BRCA2* 6174delT mutation.[1] Genomic DNA from a known heterozygous individual for each mutation was included in 96-well PCR reaction trays for each assay. Each PCR reaction had in common five microliters of the DNA preparation as template, 10 mM Tris, 50 mM KCl, 0.01% gelatin, 100 uM each dNTP, 20% sucrose, 100 uM cresol red, and 1 U Taq polymerase.

For 185delAG and 188del11 ASO assays, Exon 2 of BRCA1 was amplified in a 30 microliter PCR reaction containing 3.5 mM $MgCl_2$, 200 nM primer 2F (GAA GTT GTC ATT TTA TAA ACC TTT) and 200 nM primer 2R2 (GTA

TGT AAG GTC AAT TCT GTT C). Thermal cycling included an initial denaturation step of 94°C for 5 minutes, then 12 cycles of 10 sec denaturing at 94°C, 40 sec annealing at 68°C, and 30 sec extension at 72°C, decreasing the annealing temperature by 1°C each cycle, followed by 30 cycles of 10 sec denaturing at 94°C, 30 sec annealing at 57°C, and 30 sec extension at 72°C. Two and one half microliters of the PCR reaction were spotted onto each of four to six nylon membranes (Hybond N+, Amersham Life Science, Arlington Heights, IL) using a 96 channel device (Hydra-96, Robbins Scientific, Sunnyvale, CA). The membranes were placed in denaturing solution (1.5M NaCl, 0.5M NaOH) for one to two minutes followed by neutralizing solution (1.5M NaCl, 1M Tris pH 8.0) for one to two minutes, and cross-linked using a Stratalinker 2400 (Stratagene, La Jolla, CA). For the 185delAG ASO, oligonucleotides corresponding to the mutant sequence (ATC TTA GTG TCC CAT CT) and wild-type sequence (AAT CTT AGA GTG TCC CA) were radiolabelled as described previously[2], as were mutant (TTA GAG GTA AGT CAG CA) and wild-type (TTA GAG TGT CCC ATC TG) oligonucleotides for the 188del11 ASO. Membranes were pre-hybridized at 46°C for 1-2 hours in 5X SSPE, 5X Denhardt's Solution, and 0.5% SDS. Individual membranes were then hybridized with at least 1 X 10^6 dpm of each labeled oligonucleotide/ml of hybridization fluid for 2-12 hours at 46°C. Two hundred pmoles of unlabelled wild-type oligonucleotide were added to the mutant hybridizations and vice-versa. Membranes were washed in 2X SSC/0.1% SDS at 46°C for 10 minutes and exposed to film for 1-12 hours. Samples were scored for the mutation only if there was sufficient signal from the wild-type hybridization.

For the 5382insC allele specific PCR assay, the reaction contained 1.5 mM $MgCl_2$, 200 nM primer 20F (ATA TGA CGT GTC TGC TCC AC), 200 nM of the insertion specific primer 20Rins1 (CCT TTC TGT CCT GGG GAT T), and 100 nM primer 20R (GGG AAT CCA AAT TAC ACA GC). Thermal cycling included an initial denaturation step of 94°C for 2 minutes, then 10 cycles of 10 sec denaturing at 92°C, 20 sec annealing at 68°C, and 20 sec extension at 72°C, decreasing the annealing temperature by 1.5°C each cycle, followed by 30 cycles as for Exon 2. The reactions

were resolved on 1.8% agarose gels, producing a single band of 399-bp among wild-type individuals and both 399-bp and 143-bp bands in individuals who are heterozygous or homozygous for the 5382insC mutation.

For the 6174delT allele specific PCR assay, the reaction contained 2.0 mM $MgCl_2$, 200 nM primer TD (AAT GAT GAA TGT AGC ACG C), 200 nM of the deletion specific primer 6174delT-R (ATA CCT GGA CAG ATT TTC CC), and 100 nM primer CG (GTC TGA ATG TTC GTT ACT). Thermal cycling was as for the 5382insC assay. The reactions were resolved on a 1.8% agarose gel, producing a single band of 342-bp among wild-type individuals and both 342-bp and 143-bp bands in individuals who are heterozygous or homozygous for the 6174delT mutation.

To estimate false positive and false negative rates of the 185delAG, 5382insC, and 6174delT assays, a second DNA isolation was done on all samples initially scored as positive for any mutation, and on at least 250 samples initially scored negative for each mutation. DNA from this second purification was than assayed for the appropriate mutation. For the 185delAG mutation, an allele specific PCR assay was used containing 2.5 mM $MgCl_2$, 200 nM primer 2Fp (GGG TTG GCA GCG ATA TGT GAA AAA), 200 nM of the deletion specific primer 2R1 (TGA CTT ACC AGA TGG GAC ACT A), and 100 nM primer 2Rp (AGT GGG AGA GGC AGA GTG GAT GGA). Thermal cycling was as for the 5382insC allele specific PCR. The reactions were resolved on a 1.8 percent agarose gel, producing a single band of 527-bp among wild-type individuals and both 527-bp and 287-bp bands in individuals who are heterozygous or homozygous for the 185delAG. The assay for retesting the 5382insC mutation was the same as for the primary analysis. For the 6174delT mutation, an allele specific PCR was used as above, except that 100 nM primer 5888F (CGA AAA TTA TGG CAG GTT GTT ACG AG), 200 nM of the deletion specific primer 6174delT-F (CGA TTT TTA GCA CAG CAA GG), and 200 nM primer 6574R (GCT CTG GGT TTC TCT TAT CAA CAC GA) were used to produce a single band of 687-bp for wild-type individuals and bands of 687-bp and 420-bp for mutation

carriers. There were no false negatives and two false positives (one each for 185delAG and 6174delT). Only samples that were positive on initial and repeat testing were considered positive in the statistical analyses.

Eight negative samples and eight positive samples for each of the three mutations were reamplified and the PCR products sequenced on an ABI 377 automated sequencer and confirmed the presence or absence of the mutations.

[1] Sommer SS, Groszbach AR, Bottema, CDK. PCR amplification of specific alleles (PASA) is a general method for rapidly detecting known single-base changes. Biotechniques 1992;12:82-7.

[2] Hussussian CJ, Struewing JP, Goldstein AM, et al. Germline p16 mutations in familial melanoma. Nature Genetics 1994;8:15-21.